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NOTES

Isolation of Plasmids in *Legionella pneumophila* and *Legionella*-Like Organisms

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Agarose gel electrophoresis was employed to screen nine strains of *Legionella*-like bacteria and one strain of *Legionella pneumophila* for the presence of extrachromosomal deoxyribonucleic acid. Cryptic plasmids with molecular weights ranging from 46.6×10^6 to 59.8×10^6 were found in three of the isolates examined.

Recent reports have established that certain strains of bacteria, designated *Legionella*-like, are etiological agents of pneumonia in humans (2, 8, 10, 14). The *Legionella*-like isolates examined in this study, WIGA, TEX-KL, TATLOCK, HEBA, and five strains of the Pittsburgh pneumonia agent, possess various degrees of phenotypic or genotypic relatedness to *Legionella pneumophila*. The WIGA bacterium isolated in 1959 has been demonstrated to be phenotypically similar but genetically distinct from *L. pneumophila*, based on deoxyribonucleic acid (DNA) homology studies, and may represent a second species of *Legionella* (1). Another WIGA-like bacterium, designated TEX-KL, was obtained from postmortem lung tissue from a patient who died in Texas in early 1979. DNA relatedness studies indicate that TEX-KL may represent a third species of the genus *Legionella* (10). The 1943 isolate, TATLOCK (6), the HEBA bacterium isolated in 1959 (6), and the Pittsburgh pneumonia agent, isolated in 1979 (16) have identical biochemical, cultural, and antigenic characteristics. These strains also are phenotypically similar but genetically distinct from *L. pneumophila* (8), indicating that they may represent a fourth species of *Legionella* (7, 15). The OLDA bacterium, originally isolated in 1947, has now been shown to be a strain of *L. pneumophila*, serogroup 1 (12). Hereafter, we will refer to all isolates as *Legionella*-like, cognizant of the current taxonomic status of the OLDA isolate.

The *Legionella*-like isolates OLDA, WIGA, HEBA, TATLOCK, and TEX-KL were obtained from the Centers for Disease Control, Atlanta, Ga. The five Pittsburgh pneumonia agent strains (EK, ML, LR, JC, and GL) were kindly supplied by A. W. Pasculle (Presbyterian University Hospital, Pittsburgh, Pa.). *Pseudomonas aeruginosa* PU21 obtained from G. A. Jacoby (Massachusetts General Hospital, Boston, Mass.) contains a large 312×10^6-molecular-weight plasmid and a smaller 20×10^6-molecular-weight cryptic plasmid and was used as a control molecular weight marker. *Escherichia coli* V517 was supplied by E. M. Lederberg (Plasmid Reference Center, Stanford, Calif.). This strain was also used as a molecular weight marker for gel electrophoresis and contains eight plasmid species ranging in molecular weight from 1.36×10^6 to 35.8×10^6 (11).

Legionella-like bacteria were cultured on chemically defined medium (17) according to established parameters of growth for *L. pneumophila*. Cells from 100 ml of exponential-phase cultures were harvested by centrifugation and washed once in 10 ml of 10 mM sodium phosphate buffer (pH 7.0). Washed cells were suspended in 3.0 ml of 25% sucrose in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0), lysozyme (3.0 mg/ml) was added, and the suspension was incubated at 37°C in a shaker-incubator. After 15 to 20 min of incubation, 3.0 ml of 250 mM ethylenediaminetetraacetate (pH 8.0) was added, and the cells were chilled on ice for 5 min. Cell lysis was achieved by the addition of 1.5 ml of 20% sodium dodecyl sulfate followed immediately by incubation in a 55°C water bath for 5 min with gentle agitation. Freshly prepared 3 N NaOH was added dropwise until the pH was 12.1 to 12.4. The pH was immediately reduced to 8.5 to 9.0 with 2 M Tris-hydrochloride (pH 7.0). Denatured chromosomal DNA and cellular debris were precipitated by the addition of 1.5 ml of 20% sodium dodecyl sulfate and 3.0 ml of 5 M NaCl followed by overnight storage at 4°C. The

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following day, the lysate was centrifuged for 30 min at 17,000 × *g* at 4°C. The precipitate was discarded, and ribonuclease (2 mg/ml in distilled water, heated to 100°C for 5 min) was added to the supernatant to a final concentration of 100 μg/ml and incubated for 30 min at 37°C. Plasmid DNA was precipitated by the addition of 0.05 volume of 3 M sodium acetate and 2 volumes of cold 95% ethanol and stored at −20°C for at least 4 h. Plasmid DNA was concentrated by centrifugation for 30 min at 17,000 × *g*, and the resultant pellet was suspended in 100 to 200 μl of Tris-borate buffer. This procedure, unlike other methods attempted (1a, 3, 4, 9), permits the detection of plasmid DNA in the *Legionella* isolates. Samples were subjected to electrophoresis in 0.8 and 1% agarose (Seakem Marine Colloids, Inc., Portland, Me.), using Tris-borate running buffer and tracking dye, and stained as previously described by Meyers et al. (13). Samples were electrophoresed at 2 mA for 60 min followed by 50 mA for 90 to 210 min depending on the degree of band separation desired.

The migration patterns of purified plasmid DNA from the OLDA, WIGA, and TEX-KL isolates are shown in Fig. 1. Molecular weight estimates were determined from the relative migration rates of plasmid bands in agarose gels (Fig. 2). The OLDA strain of *L. pneumophila* contained a single covalently closed circular plasmid species, pLP3 (Fig. 1A), with an estimated molecular weight of 59.8×10^6. This was

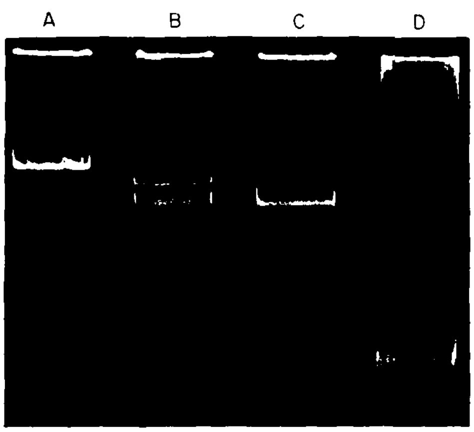

FIG. 1. *Gel electrophoresis of purified plasmids from Legionella-like bacteria. Purified DNA (30 μl) was mixed with 40 μl of tracking dye. The DNA-dye mixture (30 μl) was applied to agarose well. DNA samples were subjected to electrophoresis in 1% agarose at 2 mA for 60 min followed by 50 mA for 210 min. (A) OLDA strain of L. pneumophila, (B) WIGA isolate, (C) TEX-KL strain, (D) P. aeruginosa, PU21 control.*

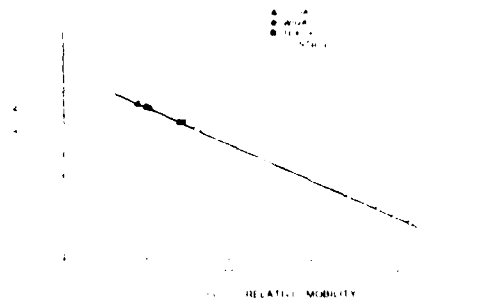

FIG. 2. *Least-squares regression analysis of plasmid DNA. Plasmid molecular weights were calculated as described by Hansen and Olsen (5). Plasmid DNA from E. coli V517 was purified by cesium chloride-ethidium bromide buoyant density centrifugation. Plasmid molecular weights of marker strain are* 35.8×10^6*,* 4.8×10^6*,* 3.7×10^6*,* 3.4×10^6*, and* 2.6×10^6*. The three smaller plasmid species of the marker strain (*2×10^6*,* 1.8×10^6*, and* 1.4×10^6*) were not retained on the gel under the stated electrophoretic conditions.*

the largest of the five plasmid isolates. The WIGA bacterium contained two plasmid species, pLB1 (molecular weight, 54.3×10^6) and pLB2 (molecular weight, 47.6×10^6) (Fig. 1B), the smaller of the two having a double band appearance. This third intermediate band is believed to be a catenated form of the smaller of the two plasmids (pLB2) in the WIGA isolate and not an open circular form of pLB2 or a third distinct plasmid species. Further evaluation of this observation by electron microscopy is in progress. The TEX-KL organism also had two plasmid species, pLK1 and pLK2, with molecular weights of 58.6×10^6 and 46.6×10^6, respectively, (Fig. 1C). The alteration of selected electrophoretic parameters shows that all plasmid isolates are unique entities. Plasmid DNA was not detected in the HEBA, TATLOCK, or five Pittsburgh pneumonia agent isolates. The failure to isolate extrachromosomal DNA from these organisms may have been due to shortcomings in our technique, and alternate methodologies may eventually establish the presence of plasmid DNA in these organisms. The small 20×10^6-molecular-weight cryptic plasmid of the control *P. aeruginosa* strain was not observed. It is possible that this culture was cured of this smaller plasmid, since extrachromosomal DNA from *E. coli* in the molecular weight range of 10×10^6 to 20×10^6 was successfully resolved by our protocol (data not shown).

The recovery of plasmid DNA from cells grown in complex medium was very low compared with recovery of plasmid material from cells grown in chemically defined medium. In addition, attempts to enhance plasmid recovery by using phenol extraction or heat-pulse (5) rather than heat elevation also resulted in lower yields of DNA. Although these plasmid species do not have high molecular weights, they appear to be highly susceptible to shear forces, which may explain our initial failures in attempting to isolate extrachromosomal DNA by conventional procedures.

The isolation of extrachromosomal DNA from members of *Legionella* is not surprising, in consideration of the ubiquitous nature of plasmid elements. These results indicate that members of this genus, like other human pathogenic microorganisms, are able to maintain stably plasmid DNA as part of their total genetic complement. The plasmid content of each proposed species is unique and may possibly be used in the future as one of several considerations for classification within the genus *Legionella*. The ability of these microorganisms to exchange genetic information with other bacteria has, to our knowledge, not been reported. However, in view of the narrow spectrum of antibiotics effective in the treatment of diseases caused by *L. pneumophila* and *Legionella*-like organisms, the acquisition of drug resistance or virulence factors by these bacteria could have serious clinical ramifications. Investigations are in progress to determine whether the plasmid isolates contribute to the virulence of these novel pathogenic microorganisms.

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